Cichotyboside, A Sesquiterpene Glycoside from *Cichorium intybus* Attenuates Carbon Tetrachloride-Induced Liver Injury in Rat

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Abstract **Aims:** *Cichorium intybus* L. is traditionally used for the management of the various liver disorders and also widely scientifically reported for hepatoprotection. The present study aims at investigating cichotyboside, bioactive sesquiterpene glycoside, from seeds for hepatoprotective effect against carbon tetrachloride (CCl₄) following isolation and characterization. **Methods:** Cichotyboside was isolated from *C. intybus* seeds and characterized by infrared spectroscopy (IR), liquid chromatography-mass spectrometry (LC/MS), and carbon 13 nuclear magnetic resonance (¹³C NMR). High-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC) have been developed for the standardization of Cichotyboside. In this study, biochemical assays, liver functional capacity restoration regarding drug metabolism, antioxidant potential, and histological staining were applied to evaluate the anti-hepatotoxic efficacy of cichotyboside on a carbon tetrachloride induced liver injury in rats. **Results:** The results showed that cichotyboside could significantly attenuate the pathological changes, increase the levels of hepatocyte repair, and regulate oxidative damage. Hepatoprotective activity of cichotyboside is indicated by its ability to decrease thiopental induced sleeping time, increased BSP clearance, reverse effect of CCl₄ on the body weight, relative liver weight, serum liver function parameters,

histopathology and activity of liver antioxidant enzymes. **Conclusion:** Cichotyboside exerts hepatoprotective activity against the CCl₄ induced toxicity, possibly by its remarkable antioxidant potential, reversing the impaired metabolisms and repairing the fatty changes in hepatocytes.

Keywords Carbon Tetrachloride, *Cichorium intybus*, Cichotyboside, Hepatoprotective, Sesquiterpene Glycoside

1. Introduction

Cichorium intybus L. commonly known as chicory and kasani belongs to family *Asteraceae*. It is a woody, erect, and perennial herbaceous plant with a fleshy taproot. *C. intybus* has been reported to possess great medicinal importance because of its marvelous phytochemical composition. *C. intybus* is useful in treating dysmenorrhoea, insomnia, splenitis, obesity, hyperlipidemia, and tachycardia [1,2]. It also possesses antiulcerogenic, anti-inflammatory, and diuretic activity [3]. Leaves and roots of *C. intybus* have strong nematocidal and antibacterial activities [4].

Liv-52 syrup, one of the bestselling liver tonics in India, is manufactured by Himalaya Healthcare Ltd. Jigreen,

manufactured by Hamdard Laboratories is a Unani polyherbal liver medication having a high market reputation. Both these formulations contain kasani as a major ingredient. Dabur research foundation has been granted with a patent for hepatoprotective composition claiming treatment of hepatitis B and E infection containing kasani [5]. *C. intybus* is used in biliary disorders like jaundice and is an ingredient of several recipes prescribed by traditional healers to cure hepato-biliary complaints [6].

The ethnobotanical studies have reported the use of leaves in curing jaundice, liver disorders, vomiting, loose motion, fever, and pleurisy [7]. Madani et al. have reported protective effects of *Silybum marianum* and *C. intybus* polyphenolic extracts on thioacetamide induced hepatotoxicity in rats [8]. A decoction of the whole plant of *C. intybus* is used for the treatment of jaundice and hepatitis in Pakistan [9]. Methanolic extract of *C. intybus* whole plant has shown hepatoprotective activity against acetaminophen induced liver damage [10]. *C. intybus* showed a protective effect against oxytetracycline induced fatty liver in rats [11]. Methanol extracts of *C. intybus*, in combination with ginger, significantly restored the carbon tetrachloride-induced (CCl_4) hepatotoxicity in rats [12]. Different extracts of *C. intybus* dried herb showed hepatoprotective activity against CCl_4 intoxication [13,14]. Hepatoprotective effect of *C. intybus* leaf extracts was reported on CCl_4 induced liver damage in rats [15,16]. Alcoholic extract *C. intybus* seed showed protection against acetaminophen and CCl_4 induced hepatic damage in rats [17,18]. *C. intybus* root and root callus extract showed a protective effect on the morphofunctional state of the liver in rats with CCl_4 induced hepatitis [19-21].

The hepatoprotective effect of *C. intybus* is reported on the whole herb, leaf, seed, and root extracts. *C. intybus* contains a number of phytochemicals such as inulin, sesquiterpene lactones, glycoside, coumarins, vitamins, sterols, flavonoids, and tannins [12,22,23]. Esculetin, a phenolic compound found in *C. intybus* have a protective effect against paracetamol and CCl_4 induced hepatic damage [24]. Zhang et al. reported in vitro hepatocytoprotective and anti-hepatitis B virus properties of cichoric acid from *C. intybus* leaves [25].

The seed of *C. intybus* afforded a sesquiterpene glycoside, cichotyboside that exhibited anti-hepatotoxic activity against CCl_4 induced toxicity by reducing serum glutamate oxaloacetic transaminase (SGOT) and serum glutamate pyruvic transaminase (SGPT) and alkaline phosphatase (ALP) level. This sesquiterpene glycoside was isolated from the methanolic extract of the *C. intybus* seeds, which has been characterized as cichotyboside {2 α ,6 β ,7 β ,15-tetrahydroxy-1(10),4(5)-diene-guaian-9 α ,12-olide-7-O- β -caffoyl-15-O- β -D-glucoside} [26]. The present study attempts isolation of cichotyboside from *C. intybus* seeds followed by characterization and authentication with the aid of high performance liquid

chromatograph (HPLC), liquid chromatography-mass spectrometry (LC/MS), and high performance thin layer chromatograph (HPTLC) techniques. Characterization of cichotyboside by HPLC, LC/MS and HPTLC was attempted for the first time to authenticate as reference standard. Hepatoprotective and antioxidant potential of cichotyboside was explored against CCl_4 induced hepatotoxicity. Estimation of liver metabolizing efficiency as a measure of thiopental induced sleeping time, and bromsulphthalein (BSP) clearance, liver function biochemical parameters, free radical scavenging ability, and histopathological changes in liver tissues was performed in order to ascertain the whole spectrum of the hepatoprotective potential of cichotyboside.

2. Materials and Methods

2.1. Drugs and Chemicals

Bromsulphthalein was procured from HiMedia Lab. Ltd., Mumbai, and thiopental was obtained as a gift sample from Neon Lab., Mumbai.

2.2. Plant Material

The seeds of *C. intybus* were collected from the local market of Bhopal, M.P., India. The seeds were identified by Dr. H. B. Singh, Scientist F, and Head, Raw Material Herbarium and Museum, NISCAIR, New Delhi, India. A voucher specimen (NISCAIR/RHMD/Consult/-2009-10/1234/47) of the seed has been retained in the department for reference purposes.

2.3. Isolation of Cichotyboside from *C. intybus*

Dry, coarse powder of *C. intybus* seed was extracted with absolute ethanol following the cold percolation method. The filtrate was concentrated to a small volume under reduced pressure using a rotary vacuum evaporator. The crude extract (yield 14.23%) was then fractionated into petroleum ether, chloroform, and methanol soluble fractions having yields 0.94, 1.12, and 7.45%, respectively. The methanol soluble fraction was subjected to a qualitative test for the presence/absence of phenolic compounds. The methanol fraction was chromatographed on Merck silica gel 60 F₂₅₄ plates. On the basis of literature search, solvent system petroleum ether: benzene: methanol was used in increasing order of polarity in order to achieve the maximum possible compounds with better separation [26]. Molish's reagent was used for the detection of spots. The 1:3:2 ratio of petroleum ether: benzene: methanol showed two well separated with R_f value 0.13 and 0.42.

2.4. Column Chromatography

Phytoconstituents present in methanol fraction were

separated by column chromatography. A column of 760 mm height, 120 mm diameter, was packed up to a height of 550 mm with a slurry of silica gel-60 (particle size 40-63 μm) in 1:3:2 ratio of petroleum ether: benzene: methanol. The methanol fraction (50 gm) was loaded on to the column, eluted with a fixed combination of 1:3:2 ratio of petroleum ether: benzene: methanol in a flow rate of 1 ml/min. The samples were collected as 10 ml elutes and simultaneously chromatographed in 1:3:2 ratio of petroleum ether: benzene: methanol. The vials 33-59 showed a single spot (R_f 0.42) and labeled as fraction C and subjected to HPLC, LC/MS, IR, ^{13}C NMR, and HPTLC analysis for identification and characterization. The dry weight of fraction C was found to be 9.44 gm, with a yield of 0.47%.

2.5. HPLC Analysis of Fraction C

The chromatographic analysis was performed on the Shimadzu LC-20AD liquid chromatography system, equipped with CAT-228-39001-38 pump, 228-393000-38 photodiode array detector working in the range 190-800 nm, a quaternary solvent delivery system, and a rheodyne injection valve fitted with a 20 μl injection loop. The data were recorded and processed with the LC solution integrated software. Baseline resolution of fraction C was obtained at $25 \pm 2^\circ\text{C}$ using stainless steel Luna column (150 mm $\times$ 4.6 mm), packed with octadecylsilane bonded to porous silica (5 μm). An isocratic solvent system consisting of methanol: water in the ratio of 80:20 (v/v) was used. The mobile phase was passed through a 0.45 PVDF filter and degassed before use. The flow rate was kept constant at 1 ml/min, and effluents were monitored at 254 nm. Fraction C showed a prominent peak in HPLC chromatogram named peak C. Sample was dissolved in methanol, filtered through a 0.45 μm membrane filter, and 20 μl of the sample was injected.

2.6. LC/MS Analysis of Fraction C

LC/MS analysis was performed at ambient temperature ($22\text{--}24^\circ\text{C}$) using Varian Inc, USA equipped with the Prostar Binary LC with 500 MS IT PDA detectors and Polaris 5-C18-50 mm $\times$ 2.0 mm column. A binary isocratic solvent system consisting of methanol and 0.1% formic acid in water in a ratio of 80:20 (v/v) was used. The flow rate was 0.2 ml/min. The operational parameters were of direct infusion mass type with ESI and APCI negative and positive mode ionization ranging from 5 to 2000 m/e . Spray chamber temperature was set at 50°C , and nebulizer gas air pressure was maintained at 35 psi. Spray shield temperature was set at ± 600 volts.

2.7. IR and ^{13}C NMR Analysis of Fraction C

The IR spectra were recorded as KBr pellets on the

Bruker spectrophotometer (USA). ^{13}C NMR data were recorded on Bruker Avance II 400 NMR spectrometer (USA) in DMSO, and chemical shifts were expressed in δ (ppm).

2.8. Authentication of Isolated Fraction C by HPTLC

HPTLC analysis was performed on a CAMAG system equipped with high pressure auto sample injector of 100 μl capacity, scanner, UV chamber, and AMAG-Linomat-5 software system for data analysis. Two mg of cichotyboside was dissolved in 5 ml of methanol and filtered through the Whatman filter paper. Two μl of cichotyboside was spotted by auto-injector as a band onto a $10 \times 10 \text{ cm}^2$ pre-coated Merck silica gel 60 F₂₅₄ plates. The plate was run up to a height of 8 cm in the saturated mobile phase of petroleum ether: benzene: methanol in the ratio of 1:3:2 (v/v). Plates were removed, air dried after development, and finally dried with the aid of drier. The scanning of the plate was performed at 254 nm, and the data was recorded.

2.9. Assessment of Hepatoprotective Activity of Cichotyboside

2.9.1. Test Animals

Laboratory bred Wistar albino rats of either sex weighing between 150-200 gm were maintained under standard laboratory conditions at $22 \pm 2^\circ\text{C}$, relative humidity $60 \pm 5\%$, and photoperiod of 12 hr-dark and light. Commercial pellet diet (Hindustan Lever, India) and water were provided *ad-libitum*. Animals were not allowed to access water and food before and after 1 hr of dosing. All the experimental interventions were carried out at the same time of day in order to avoid diurnal variation. Institutional Animal Ethics Committee approval was obtained before carrying out animal experiments following the guideline of Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi.

2.9.2. Treatment protocol

One lower dose of 25 mg/kg and one higher dose of 100 mg/kg, along with a medium dose of 50 mg/kg, were selected. Animals were randomly divided into six groups, with 12 rats in each. Group I and III-VI were treated as vehicle control (normal saline 0.2 ml/100 gm, p.o), positive control (silymarin, p.o), and three different doses of cichotyboside (25, 50 and 100 mg/kg, p.o) continuously for 14 days. Animals of groups (I-VI) were treated with CCl_4 (1 ml/kg, i.p., of a mixture of 1:1 in liquid paraffin) 2 hr after drug treatment on the 7th day, and afterward on alternate days for further 7 days. On the 14th day, 2 hr after drug treatment, 6 animals from each group were used to determine thiopental induced sleeping

time. The remaining 6 animals from each group were used for biochemical analysis of serum for marker enzymes, BSP clearance, free radical scavenging ability of the liver, and histopathological study of the liver.

Body weights of all the animals were recorded on 1st, 7th, and 14th day before sacrifice. The estimation of CCl₄ induced prolongation of thiopental sodium (40 mg/kg, i.p) sleeping time as a measure of liver metabolizing efficiency was done by following the method of Singh et al. [27]. Percentage hepatoprotection in terms of in vitro BSP clearance by liver slices was calculated following the method of Ranjan and Subrahmanyam [28]. Six animals of each group were anesthetized by light ether anesthesia, and blood was withdrawn by the intracardiac puncture. Blood was kept at room temperature to coagulate for 30 min, and serum was separated by centrifugation at 3000 rpm for 5 min. The serum was used to estimate serum SGOT, SGPT, ALP, cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), triglyceride, total and direct bilirubin, protein, and albumin as per the method described in diagnostic kits (Aspen Diagnostic Pvt. Ltd., India). The liver, kidney, spleen, and heart were harvested, washed in normal saline, blotted in filter paper, and weighed. Each liver was cut into three portions. Three slices of 60 mg each were cut and weighed from the first part that was used for the BSP clearance test. Liver slices were placed in media (KCl:10 mM, MgSO₄: 1 mM, NaCl: 1 mM in phosphate buffer) containing 30 µg BSP/ml and incubated at 38°C. An aliquot of reaction mixture was analyzed after 10, 20 and 30 minutes using spectrophotometer at 580 nm to determine the BSP concentration in the media [28]. The second part of the liver was used for the estimation of free radical scavenging ability for glutathione [29], lipid peroxidase [30], and superoxide dismutase [31]. The third part of the liver was preserved in a 10% formalin solution for the histopathological assessment of liver damage. Hematoxylin and eosin staining of liver tissues and the permanent tissue slides were prepared by the following method of Nanji et al. [32].

2.10. Statistical Analysis

The results were expressed in terms of Mean ± SEM. Experimental data were analyzed using one way ANOVA followed by Turkey-Kramer multiple comparisons using the InStat-3 graph pad prism version. Differences between the values of the groups compared were considered significant at $P < 0.05$.

3. Results

3.1. Characterization of Cichotyboside

The isolated fraction C was obtained as a viscous brown solid mass, which showed a prominent peak having a retention time of 6.906 min (Peak C) in HPLC analysis. LC/MS analysis of fraction C showed one well defined peak with a retention time of 7.952 min, designated as Peak 1A in LC/MS chromatogram. The Peak 1A showed m/z at 472.2 (M^+), 454.5, 308.5, 280.4, 264.7, 262.2, 220.3, 202.7 and 163.3. IR spectrum of fraction C indicated the presence of -COOH and alcoholic group (3530.73 cm^{-1}), γ -lactone, ester linkage, and carbonyl group (1654.19 cm^{-1}) and double bond (1574.69 and 714.48 cm^{-1}).

The ¹³C NMR spectrum of fraction C exhibited the peaks at δ 133.13 (C-1), δ 196.51 (C- 2), δ 40.27 (C-3), δ 136.26 (C-4), δ 132.20 (C-5), δ 77.25 (C-6), δ 39.36 (C- 7), δ 78.71 (C-8), δ 77.36 (C- 9), δ 141.46 (C- 10), δ 39.57 (C-11), δ 177.89 (C-12), δ 39.78 (C-13), δ 39.99 (C-14), δ 127.83 (C-1'), δ 127.92 (C- 2'), δ 128.02 (C- 3'), δ 128.13 (C- 4'), δ 128.17 (C- 5'), δ 128.22 (C-6'), δ 143.09 (C- 7'), δ 129.08 (C-8') and δ 155.46 (C- 9'). These peaks are well comparable with mass, IR, and ¹³C NMR data reported by Ahmed et al. and confirm the isolation of cichotyboside. Cichotyboside was further subjected to HPTLC analysis using solvent system petroleum ether: benzene: methanol (1:3:2), which showed the major spot with R_f value 0.45 having the area 97.44% and another spot with R_f value 0.72 having the area 2.68% (Figure 1, 2, 3, 4, 5 and 6).

Hepatoprotective activity

Peak C showed retention time at 6.906 min.

Figure 1. HPLC chromatogram of fraction C in mobile phase methanol: water (80:20) scanned at 254 nm.

The peak 1A showed m/z at 472.2 (M^+), 454.5, 308.5, 280.4, 264.7, 262.2, 220.3, 202.7 and 163.3.

Figure 2. LC/MS chromatogram of Fraction C.

Figure 3. Probable fragmentation pattern of fraction C.

IR spectrum of fraction C indicated the presence of -COOH and alcoholic group (3530.73 cm^{-1}), γ -lactone, ester linkage and carbonyl group (1654.19 cm^{-1}) and double bond (1574.69 and 714.48 cm^{-1}).

Figure 4. IR spectrum of fraction C.

C-2 exhibited the peaks at $\delta 133.13$ (C-1), $\delta 196.51$ (C-2), $\delta 40.27$ (C-3), $\delta 136.26$ (C-4), $\delta 132.20$ (C-5), $\delta 77.25$ (C-6), $\delta 39.36$ (C-7), $\delta 78.71$ (C-8), $\delta 77.36$ (C-9), $\delta 141.46$ (C-10), $\delta 39.57$ (C-11), $\delta 177.89$ (C-12), $\delta 39.78$ (C-13), $\delta 39.99$ (C-14), $\delta 127.83$ (C-1'), $\delta 127.92$ (C-2'), $\delta 128.02$ (C-3'), $\delta 128.13$ (C-4'), $\delta 128.17$ (C-5'), $\delta 128.22$ (C-6'), $\delta 143.09$ (C-7'), $\delta 129.08$ (C-8') and $\delta 155.46$ (C-9').

Figure 5. ^{13}C NMR spectrum of fraction C.

Cichotyboside showed R_f 0.45 and 0.72 having area percent 97.44 and 2.68 respectively.

Figure 6. HPTLC chromatogram of cichotyboside in solvent system petroleum ether: /benzene: methanol (1:3:2) scanned at 254 nm.

Table 1. Effect of cichotyboside on body weight of CCl_4 treated rats

Treatment mg/kg, p.o.	Body weight in gm			% Change in body weight	
	0 day	7 th day	14 th day	7 th day	14 th day
Vehicle control	176.24 ± 11.51	183.75 ± 8.56	188.25 ± 10.51	4.26	6.81
CCl_4 (1 ml/kg)	191.21 ± 7.56	196.96 ± 8.12	181.52 ± 7.89	3.01	-5.06
Silymarin (20)	168.76 ± 7.55	172.51 ± 15.05	176.48 ± 12.45	2.22	4.57
Cichotyboside (25)	154.26 ± 1.64	157.25 ± 13.33	149.52 ± 10.36	1.94	-3.07
Cichotyboside (50)	151.51 ± 1.66	154.52 ± 10.58	150.50 ± 11.68	1.98	-0.66
Cichotyboside (100)	187.52 ± 8.66	195.56 ± 10.40	197.52 ± 8.81	4.28	5.33

Data represented as Mean ± SEM of 6 animals in each group.

3.2. Body Weight and Relative Organs Weight

Post-treatment with silymarin and cichotyboside (100 mg/kg) resulted in body weight gain by 4.57 and 5.33% in comparison to the decreased body weight (5.06%) in CCl_4 treated group. Rats administered with CCl_4 showed an increase (3.89 ± 0.17) in relative liver weights, whereas silymarin and cichotyboside (50 and 100 mg/kg) revealed significant decrease ($P < 0.05$ and 0.001) in the relative

liver weight. Cichotyboside has a non-significant effect on the relative kidney, spleen, and heart weight (Tables 1 and 2).

3.3. Thiopental Induced Sleeping Time

Sleeping time (195.51 ± 4.95 min) was significantly increased in CCl_4 intoxicated rats compared to vehicle control group (61.26 ± 2.64 min). Silymarin and

cichotyboside resulted in an extremely significant decrease ($P<0.001$) in thiopental induced sleeping time. The percentage hepatoprotection was 65.67, 83.58, and 88.80% at 25, 50, and 100 mg/kg doses of cichotyboside in respect to silymarin (97.76%) (Table 3).

3.4. Bromsulphthalein Uptake by Liver Slices

Cichotyboside showed dose-dependent hepatoprotection of 72.26, 80.95, and 83.33% respectively after 30 min of incubation whereas, and silymarin showed a hepatoprotection of 86.90% at 30 min (Figure 7).

Table 2. Effect of cichotyboside on relative organs weight of CCl_4 treated rats

Treatment (mg/kg, p.o.)	Organ weight/100 gm of body weight in gm			
	Liver	Kidney	Spleen	Heart
Vehicle control	3.20 ± 0.13	0.70 ± 0.05	0.21 ± 0.04	0.59 ± 0.08
CCl_4 (1 ml/kg)	3.89 ± 0.17^b	0.79 ± 0.05^{ns}	0.27 ± 0.05^{ns}	0.68 ± 0.08^{ns}
Silymarin (20)	$3.28 \pm 0.02^{*,ns}$	$0.68 \pm 0.08^{ns,ns}$	$0.23 \pm 0.01^{ns,ns}$	$0.58 \pm 0.01^{ns,ns}$
Cichotyboside (25)	$3.46 \pm 0.12^{ns,a}$	$0.76 \pm 0.04^{ns,ns}$	$0.24 \pm 0.01^{ns,ns}$	$0.65 \pm 0.02^{ns,ns}$
Cichotyboside (50)	$3.40 \pm 0.18^{*,a}$	$0.73 \pm 0.06^{ns,ns}$	$0.23 \pm 0.03^{ns,ns}$	$0.63 \pm 0.01^{ns,ns}$
Cichotyboside (100)	$3.08 \pm 0.14^{***,ns}$	$0.71 \pm 0.06^{ns,ns}$	$0.20 \pm 0.07^{ns,ns}$	$0.60 \pm 0.06^{ns,ns}$

Data represented as Mean $\pm$ SEM of 6 animals in each group. $^*P<0.05$, $^{***}P<0.001$ compared to the negative control (CCl_4 treated) group. $^aP<0.05$, $^bP<0.01$ compared to the vehicle control group, and ns = not significant.

Table 3. Hepatoprotective efficiency of cichotyboside related to restoration of thiopental metabolism associated effect on sleeping time against CCl_4 induced liver damage on rats

Treatment (mg/kg, p.o.)	Sleeping time in min	% Hepatoprotection
Vehicle control	61.26 ± 2.64	-
CCl_4 (1 ml/kg)	195.51 ± 4.95^c	-
Silymarin (20)	$64.28 \pm 3.51^{***,ns}$	97.76
Cichotyboside (25)	$107.83 \pm 4.03^{***,b}$	65.67
Cichotyboside (50)	$83.65 \pm 5.16^{***,ns}$	83.58
Cichotyboside (100)	$76.39 \pm 4.46^{***,ns}$	88.80

Data represented as Mean $\pm$ SEM of 6 animals in each group. $^{***}P<0.001$ compared to the negative control (CCl_4 treated) group. $^bP<0.01$, $^cP<0.001$ compared to the vehicle control group, and ns = not significant.

Figure 7. Hepatoprotective effect of cichotyboside on BSP uptake of CCl_4 treated rat liver slices.

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Table 4. Hepatoprotective effect of cichotyboside associated serum biochemical parameters of CCl₄ treated rats

Treatment (mg/kg, p.o.)	SGOT IU/l	SGPT IU/l	ALP U/l	Cholesterol mg/dl	Triglyceride mg/dl	HDL mg/dl	LDL mg/dl	Bilirubin mg/dl		Protein gm/dl	Albumin gm/dl
								Total	Direct		
Vehicle control	46.38 ± 2.78	39.08 ± 2.54	47.26 ± 2.18	126.33 ± 5.46	126.49 ± 7.24	47.55 ± 3.39	53.48 ± 3.29	1.52 ± 0.07	0.63 ± 0.02	6.80 ± 0.33	4.97 ± 0.21
CCl ₄ (1 ml/kg)	92.65 ± 5.01 ^c	104.81 ± 6.51 ^c	132.65 ± 6.98 ^c	189.63 ± 10.65 ^c	261.16 ± 5.86 ^c	22.64 ± 2.09 ^c	114.75 ± 5.98 ^c	2.12 ± 0.12 ^a	2.06 ± 0.26 ^c	4.86 ± 0.17 ^b	3.68 ± 0.11 ^a
Silymarin (20)	40.88 ± 2.14 ^{***,ns}	53.63 ± 3.37 ^{***,ns}	49.11 ± 6.16 ^{***,ns}	134.56 ± 6.03 ^{*,ns}	178.56 ± 4.98 ^{***,ns}	48.59 ± 2.12 ^{***,ns}	50.25 ± 2.19 ^{***,ns}	1.56 ± 0.12 ^{ns,ns}	0.96 ± 0.01 ^{***,ns}	6.04 ± 0.25 ^{*,ns}	4.39 ± 0.15 ^{*,ns}
Cichotyboside (25)	48.63 ± 5.02 ^{***,ns}	49.50 ± 2.91 ^{***,ns}	86.73 ± 2.78 ^{***,a}	159.33 ± 8.67 ^{ns,a}	197.36 ± 4.98 ^{***,a}	32.67 ± 2.19 ^{ns,a}	87.19 ± 4.52 ^{***,a}	2.06 ± 0.11 ^{ns,a}	1.09 ± 0.05 ^{***,a}	5.97 ± 0.16 ^{*,a}	3.92 ± 0.12 ^{ns,a}
Cichotyboside (50)	40.36 ± 3.54 ^{***,ns}	44.96 ± 5.45 ^{***,ns}	73.58 ± 6.95 ^{***,a}	151.61 ± 11.74 ^{*,ns}	183.47 ± 8.93 ^{***,a}	35.42 ± 3.17 ^{*,ns}	79.49 ± 4.28 ^{***,ns}	1.93 ± 0.33 ^{ns,a}	1.05 ± 0.03 ^{***,a}	6.14 ± 0.23 ^{**,ns}	4.16 ± 0.13 ^{ns,a}
Cichotyboside (100)	38.19 ± 3.80 ^{***,ns}	41.65 ± 4.86 ^{***,ns}	59.34 ± 2.44 ^{***,ns}	143.95 ± 6.90 ^{**,ns}	169.66 ± 7.58 ^{***,ns}	42.56 ± 3.64 ^{***,ns}	67.45 ± 4.15 ^{***,ns}	1.87 ± 0.09 ^{ns,a}	0.82 ± 0.14 ^{***,ns}	6.23 ± 0.27 ^{**,ns}	4.27 ± 0.13 ^{ns,ns}

Data represented as Mean ± SEM of 6 animals in each group. *P<0.05, **P<0.01, ***P<0.001 compared to the negative control (CCl₄ treated) group. ^aP<0.05, ^bP<0.01, ^cP<0.001 compared to the vehicle control group, and ns = not significant.

Table 5. Effect of cichotyboside on free radical scavenging ability of CCl₄ treated rat liver

Treatment (mg/kg, p.o.)	Glutathione $\mu\text{g/gm}$ of liver	Lipid peroxidase nmol/gm of protein	Superoxide dismutase Unit/mg of protein
Vehicle control	21.05 $\pm$ 1.83	3.88 $\pm$ 0.71	6.24 $\pm$ 0.76
CCl ₄ (1 ml/kg)	5.61 $\pm$ 0.69 ^c	21.38 $\pm$ 1.69 ^c	3.13 $\pm$ 0.59 ^a
Silymarin (20)	19.61 $\pm$ 1.57 ^{***,ns}	4.65 $\pm$ 0.58 ^{***,ns}	6.06 $\pm$ 0.71 ^{*,ns}
Cichotyboside (25)	8.30 $\pm$ 0.71 ^{ns,c}	4.38 $\pm$ 0.77 ^{***,ns}	3.33 $\pm$ 0.58 ^{ns,a}
Cichotyboside (50)	11.18 $\pm$ 0.97 ^{*,a}	4.32 $\pm$ 0.64 ^{***,ns}	4.56 $\pm$ 0.62 ^{ns,a}
Cichotyboside (100)	14.32 $\pm$ 1.23 ^{***,ns}	4.12 $\pm$ 0.64 ^{***,ns}	5.59 $\pm$ 0.48 ^{*,ns}

Data represented as Mean $\pm$ SEM of 6 animals in each group. *P<0.05, ***P<0.001 compared to the negative control (CCl₄ treated) group. ^aP<0.05, ^cP<0.001 compared to the vehicle control group, and ns = not significant.

3.5. Serum Biochemical Parameters

The outcome of biochemical parameters revealed a severe increase in the levels of SGOT, SGPT, ALP, cholesterol, triglyceride, LDL, total and direct bilirubin in plasma. In contrast, the level of HDL, protein, and albumin was decreased considerably in CCl₄ intoxicated animals. Cichotyboside dose-dependently showed a significant decrease (P< 0.01 to 0.001) in serum SGOT, SGPT, ALP, triglyceride, LDL, and cholesterol level, whereas, serum HDL and protein level was significantly increased (P<0.05 and 0.001). Cichotyboside has a non-significant effect on the normalization of total bilirubin while showed a highly significant decrease (P<0.001) in direct bilirubin (Table 4).

3.6. Free Radical Scavenging Ability of Liver

Treatment with cichotyboside showed a significant increase (P<0.05-0.001) in glutathione and superoxide

dismutase levels compared to the decreased level in the CCl₄ group. Cichotyboside dose-dependently caused an extremely significant decrease (P<0.001) in the liver lipid peroxidase level (Table 5).

3.7. Histopathology

Histopathological assessment of hematoxylin-eosin stained liver sections showed a concentrically arranged normal hepatocytes around the central vein and portal tracts in the vehicle control rat liver tissue. CCl₄ intoxicated liver hepatocytes showed neutrophilic infiltration, sinusoidal dilatation, zonal necrosis, vacuolar degeneration, and microvesicular fatty changes. Liver tissue of silymarin treated group showed a small area of focal necrosis and slightly altered hepatic parenchyma. Cichotyboside (100 mg/kg) treatment showed sinusoidal dilatation, very little focal coagulative necrosis, and nearly normalized hepatocytes (Figure 8a-f).

Figure 8. Assessment of CCl_4 induced hepatotoxicity by histopathology in haematoxylin-eosin stained liver sections.

(a) The liver of control animal showed normal cellular architecture with distinct hepatocyte; (b) CCl_4 intoxicated liver tissue showed neutrophilic infiltration, sinusoidal dilatation, zonal necrosis, extensive diffuse vacuolar degeneration engorged with blood and microvesicular fatty changes in hepatocytes; (c) Silymarin treated liver tissue showed small area of focal necrosis and slightly altered hepatic parenchyma; (d) Cichotyboside (25 mg/kg) pretreatment showed coagulative, lobular and centrilobular necrosis along with sinusoidal dilatation; (e) Cichotyboside (50 mg/kg) pretreatment showed focal coagulative necrosis, sinusoidal dilatation and altered hepatic parenchyma; (f) Cichotyboside (100 mg/kg) pretreatment showed sinusoidal dilatation, very little focal coagulative necrosis with nearly normalized hepatocytes.

4. Discussion

C. intybus is a traditionally well-acclaimed hepatoprotective plant having usability in treating liver damage. Whole plant, leaf, root, and seeds extracts have been scientifically explored for hepatoprotection. Leaves are reported to contain hepatoprotective phytoconstituents

esculetin and chicoric acid [33]. Esculetin is reported to reduce oxidative stress by decreasing liver cell swelling, necrosis and leukocyte infiltration [34,35]. Ahmed et al. isolated cichotyboside from *C. intybus* seeds, and reported hepatoprotective activity by estimating SGOT, SGPT, and ALP only following CCl_4 induced hepatotoxicity on rats

[26]. This study attempts the isolation of the promising hepatoprotective bioactive compound cichotyboside from *C. intybus* seeds and standardization by spectral analysis and further characterization by HPLC and HPTLC. This study is the first to report HPLC and HPTLC characterization of cichotyboside for future identification and reference standard purposes, which will help in the identification and also for reference standard purposes. This study attempts to evaluate the effect of cichotyboside on liver damage reversibility, antioxidant potential, metabolic functionality, and histopathology.

CCl_4 induced hepatotoxicity is an authentic experimental model to evaluate hepatoprotective agents. The cytochrome P_{450} system biotransforms CCl_4 to an active hepatotoxic metabolite, trichloromethyl radical. Trichloromethyl radical covalently binds to the cell protein, starting a chain of reactions eventually leading to cell and endoplasmic reticulum membrane lipid peroxidation. Lipid peroxidation leads to the generation of malondialdehyde that damages the cytoplasmic membrane. The peroxidative products cause hypoperfusion of the membrane, resulting in the escape of cytosolic enzymes in the blood [36]. A significant loss of body weight was observed in the negative control group compared to the control group accompanied with reduced food consumption. An increase in liver weight is common in the case of CCl_4 intoxication due to fatty changes [37].

Cichotyboside treatment showed a significant reversal towards the dose-dependent gain of body weight and relative liver weight suggesting normalization of CCl_4 toxicity towards the regeneration of hepatocytes. The liver plays a pivotal role in the metabolism and elimination of substances from the portal circulation by two distinct pathways via cytochrome p-450 and GSH-peroxidase. Animals with intoxicated liver show prolonged sleeping duration while treated with barbiturates due to damage of hepatic microsomal drug metabolizing enzymes. Thiopental induced sleeping time increases as the enzyme responsible for its metabolism is reduced in liver damage [38]. Cichotyboside showed a protective effect, as evidenced by decrease in sleeping duration, indicating restoration of barbiturate metabolizing capacity may be through modulation of hepatic biotransformation. Cichotyboside at all three doses has significantly improved the BSP clearance capacity of the damaged liver, maintaining phase II drug metabolism capacity, enabling to excrete the dye and, in turn, signifying less damage to the hepatic cells.

Hepatotoxin CCl_4 gets converted into CCl_3O^- by liver enzymes that attack the unsaturated fatty acids of the cell membrane. This consequently gives rise to lipid peroxidation altering the functional integrity of hepatocyte mitochondria and, ultimately, liver damage. Toxicants disbalances synthesis and metabolism of triglycerides, cholesterol, and lipoproteins by damaging the hepatocytes. The serum liver function marker enzymes like SGOT,

SGPT, and ALP levels are elevated [36]. Serum triglyceride and bilirubin (total and direct) levels elevated, whereas serum HDL, total protein, and albumin levels decreased. The increased triglyceride content in the blood indicates fatty degeneration of the liver. Two prime manifestations of liver failure are the deposition of triglycerides leading to fatty liver and disturbance in cholesterol synthesis rate that occurs following disturbance in lipid metabolism. These are common manifestations of chronic alcoholism featuring disordered hepatic lipid metabolism. Ahmed et al. reported that 50 mg/kg (p.o.) dose of cichotyboside is relatively safe and showed maximum hepatoprotective effect against CCl_4 induced hepatotoxicity in Wistar albino rats [26]. Based on the three dose 25, 50 and 100 mg/kg was evaluated in vivo. Cichotyboside treatment showed a near normal level of liver marker enzymes exhibiting hepatoprotective effect. This decrease may be due to cichotyboside induced enhancement in cell integrity and, as a consequence, reduced leakage of enzymes into the bloodstream.

Cichotyboside treatment demonstrated a considerable reduction in serum triglyceride and cholesterol levels indicating normalization of synthesis rate. Interference of hepatic bilirubin uptake can produce a mild unconjugated hyperbilirubinemia [39]. Liver cell injury impedes hepatic uptake of unconjugated bilirubin resulting in Hyperbilirubinemia [40]. Cichotyboside treatment has significantly reduced the total and direct bilirubin levels in serum. Reduced serum total proteins level in the CCl_4 treated rats directly correlates with hepatopathy severity. Hypoalbuminemia is mostly associated with advanced chronic liver diseases, which is correlated with the severity of cellular dysfunction. Cichotyboside normalizes the serum protein concentration of CCl_4 intoxicated rats, demonstrating a beneficial response on cellular macromolecule regeneration.

Hepatocytes are defended from oxidative damage by non-enzymatic and enzymatic systems like glutathione peroxidase, superoxide dismutase, and catalase. Oxidative stress induces an imbalance in the formation and removal of free radicals giving rise to the production of reactive oxygen species like superoxide, hydrogen peroxide, and hydroxyl radicals. In severe oxidative stress, these oxidative compounds interact with cellular lipid, protein, DNA, and plasma membrane leading to pathological implications. Lipid peroxidation is considered one of the major biochemical hallmarks of CCl_4 -induced hepatotoxicity. An increase in glutathione activity in cichotyboside treated groups indicated the restoration of vital molecules and reversal of peroxidative damage.

The whole herb, leaf, seed, and root of *C. intybus* is being reported to have hepatoprotective potential earlier [12,22,23]. Phytoconstituents like Esculetin and cichoric acid is also reported for potential protective effect against paracetamol, CCl_4 and anti-hepatitis B virus induced hepatic damage [24,25]. Cichotyboside is the most

promising phytochemical [26] which surely needed further evaluation. Comprehensive hepatoprotective assessment of cichotyboside in this study reveals its multifaceted beneficial effect as a protective from oxidative stress induced hepatocyte damage. Cichotyboside showed a significant reduction in lipid peroxidation, indicating its antioxidant activity further supported by the restoration of superoxide dismutase in the liver. Cichotyboside may have maintained cellular defense mechanisms by preventing cell membrane oxidation. Cichotyboside treatment reversed neutrophilic infiltration, necrosis, vacuolar degeneration, and fatty changes in hepatocytes. Hepatoprotective activity of cichotyboside can be attributed to the preservation of hepatocyte membrane structural integrity by protecting from breakage by the reactive metabolites. Plants containing sesquiterpene glycoside constituents are reported to have hepatoprotective activity against D-galactosamine, CCl₄, and acute immunological liver injury [41-43].

5. Conclusion

The mass, IR, and ¹³C NMR data confirms the isolation of cichotyboside compared to previous report. This is first report of cichotyboside LC/MS with a well-defined peak that showed mass fragmentation pattern signifying breakdown of cichotyboside. All the study parameter outcome substantiates hepatoprotective potential of cichotyboside against CCl₄ -induced liver damage. Hepatoprotective activity of cichotyboside may be due to its sesquiterpene glycoside structure, which prevents hepatocyte plasma membrane alteration and promote repair. Histopathological evaluation revealed that cichotyboside reduced oxidative stress in rat liver characterized by decreased fatty changes, leukocyte infiltration, and necrosis. Cichotyboside plays a preventative role in free radical generated hepatotoxicity by reversing metabolic functionality and cell damage. This study provided new insights into the mechanism of the hepatoprotective effect of cichotyboside from *C. intybus*, A well reputed plant used for the treatment of liver disease. The developed HPLC and HPTLC methods can be used for the quality control analysis of *C. intybus* and can also be explored for the pharmacokinetic and pharmacodynamic analysis in the future.

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Declaration of Competing Interest

The authors have no conflicts to declare.

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